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Agents Against Estrogen-Independent Breast Cancer Cells

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)
We have investigated the effect of novel selective retinoid-related molecules that induce apoptosis in breast cancer cells on IKK/NFκB activity. We identified one retinoid antagonist that elicited a strong inhibition of IKK in the ER-negative cell line MDA-MB-468. Other retinoid analogs were not as potent IKK inhibitors in intact cells, although they exerted a significant inhibition of IKK *in vitro* and a strong inhibition of cell proliferation that correlated with the induction of apoptosis in ER-negative cells. Our data observed in breast cancer cells as well as in cells obtained from other type of human tumors indicate that the inhibition of IKK/NFκB activity is critical for the induction of apoptosis by the retinoid antagonist, but not by other retinoid analogs. Our findings with non-retinoid analogs known to inhibit IKK and a non-pharmacological approach to block IKK/NFκB signaling, indicate that inhibition of this pathway is sufficient to induce cell death. Therefore, inhibitors of IKK could serve as promising new anticancer agents, either as a stand-alone therapy or in combination therapies with other anticancer approaches. In this respect, it is noteworthy that inhibitors of IKK sensitize tumor cells to the anticancer activity of certain chemotherapeutic drugs.

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INTRODUCTION

Natural and synthetic retinoids inhibit the proliferation of several cancer cell lines, including breast cancer cells (BCCs). However, upon progression to estrogen-independent growth, BCCs become resistant to the antiproliferative activity of retinoic acid (RA) and other derivatives. We have recently found a novel series of retinoid related molecules (RRMs) that are selective for RAR γ , which are potent inducers of apoptosis in several cancer cell lines. In addition, a retinoid antagonist MX781 induces apoptosis in estrogen-dependent as well as estrogen-independent BCCs and is effective against solid tumors derived from a human BCC in an animal model (Fanjul et al., 1998). Aberrant expression of NF κ B has been associated with oncogenesis and carcinogenesis (Mayo and Baldwin, 2000; Karin et al., 2002), and constitutively high levels of NF κ B activity have been detected in tumor cells. Particularly high activity was found in breast cancer cells and this activity increased in more advanced tumors (Nakshatri et al., 1997; Sovak et al., 1997), although other studies have shown that most human breast cancer cells have NF κ B activated independently of the hormonal status (Cogswell et al., 2000). Activation of NF κ B requires the phosphorylation of I κ B by IKKs (DiDonato et al., 1997), which triggers proteasome-dependent I κ B degradation and subsequent translocation of free NF κ B subunits into the nucleus. Inhibition of NF κ B by means of chemical inhibitors or microinjection of I κ B protein or an anti-c-Rel antibody was shown to induce apoptosis in B cells (Wu et al., 1996), while NF κ B expression prevented TNF α -induced apoptosis (Beg and Baltimore, 1999; Wang et al., 1996; Van Antwerp et al., 1996; Liu et al., 1996). More recently, it has been shown that inhibition of NF κ B enhances the antitumor activity of TNF α and a camptothecin analog in nude mice (Wang et al., 1999). Therefore, compounds that block NF κ B activation pathways, for example by inhibiting IKK, could serve as novel anticancer agents and, as recently suggested, might be particularly useful for the treatment of ER negative breast cancer (Biswas et al., 2000; Biswas et al., 2001).

BODY

Synthetic retinoids inhibit the proliferation of BCCs. The effect of several retinoids on the proliferation of different breast cancer cell lines has been investigated. Different concentrations of the retinoids were tested and cell proliferation was measured using a MTT assay after various incubation times. Cells grown in the absence of retinoids served as control. Figure 1A shows the results obtained with some representative compounds on MCF-7 (ER+) and MDA-MB-468 (ER-) cells, treated for increasing days in 0.5 or 10% fetal bovine serum (FBS) with 6 μ M of the indicated compounds. A very potent antiproliferative activity was found with the RAR γ -selective compounds MX3350-1 and MX2870-1, as well as 4-HPR, while no significant effect was observed with RA after short periods of incubation. RA, however, elicited strong antiproliferative effect after longer periods of incubation in MCF-7 cells (not shown). Interestingly, a novel retinoid antagonist (MX781) also showed strong antiproliferative activity against these cell lines. The antiproliferative activity of the retinoids was significantly higher when cells were treated in 0.5% FBS, which is attributable in part to a higher accumulation of retinoids in the intracellular space (our unpublished observations). Similar results were obtained in other BCCs analyzed, including MDA-MB-231, MDA-MB-435, and T47D (data not shown).

We investigated whether the antiproliferative activity of the selective RRM correlated with the induction of apoptosis. The appearance of a characteristic DNA ladder pattern in

retinoid-treated cells clearly observed when MCF-7 cells were treated with 6 μ M MX2870-1 and 4-HPR in 0.5% FBS for 24 hours (data not shown). To quantitate this effect, we used a Cell Death Detection kit (Roche) that measures the amount of histone associated DNA fragments present in the cytosol. A strong induction of apoptosis was observed when cells were treated overnight with 6 μ M MX2870-1, MX781, or 4-HPR (Fig. 1B).

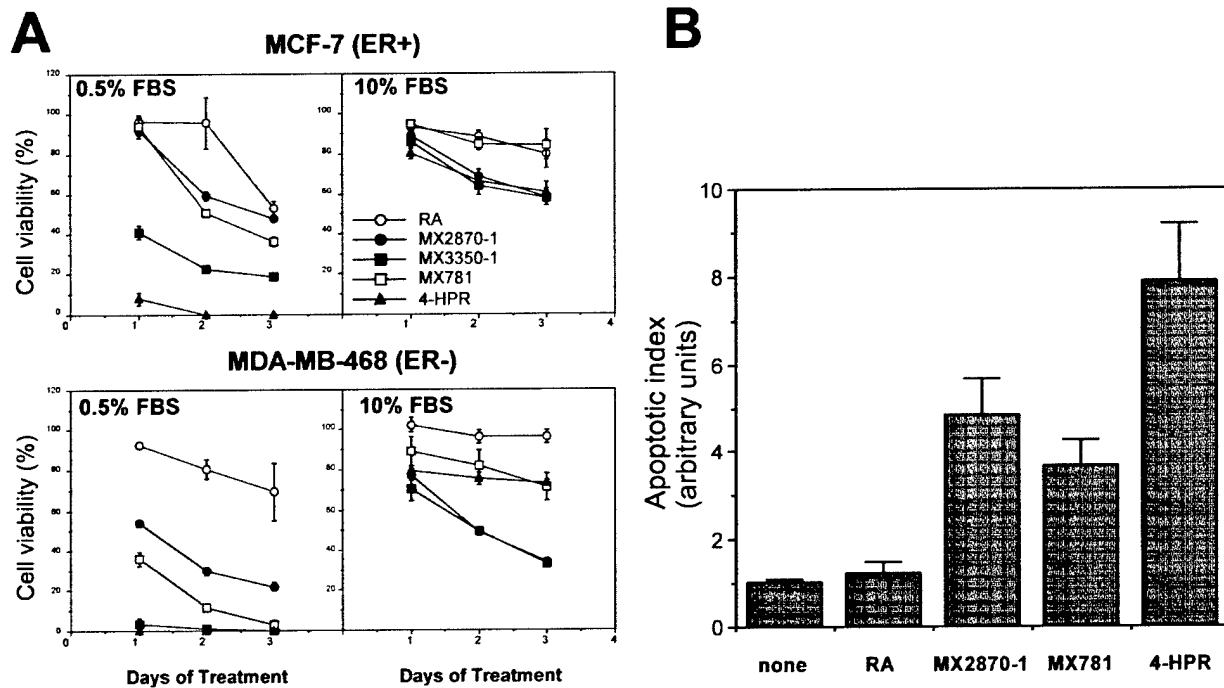


Figure 1. Selective RRM inhibition of cell growth and induction of apoptosis in breast cancer cells. (A) MCF-7 and MDA-MB-468 cells were seeded in 96 well plates (3,000 cells in 100 μ l per well). After cells were attached, the medium was changed to 0.5% FBS where indicated and retinoid treatment was initiated by adding 10 μ l of the appropriate dilution (in culture medium). Cell proliferation was measured by MTT after 1, 2, or 3 days of RRM exposure and the percentage of cell viability with respect to control untreated cells is shown. (B) Induction of DNA fragmentation by selective RRM. MCF-7 cells were treated with 6 μ M of the indicated compounds in medium containing 0.5% FBS. After 16 hours, cytosol extracts were obtained and the amount of DNA fragments was calculated using a Cell Death Detection ELISA, following manufacturer's instructions.

Inhibition of IKK/NF κ B signaling induces cell death in cancer cells. When analyzing the effect of a series of synthetic retinoid derivatives on the DNA binding activity of various transcription factors, we found that certain compounds that induced apoptosis inhibited the binding of NF κ B to DNA without affecting the levels of NF κ B proteins (data not shown). Noteworthy was the effect of the antagonist RRM MX781, which inhibited TNF α -induced NF κ B DNA binding activity in various prostate and lung cancer cell lines, while the effect of the RAR γ -selective compounds was cell type specific (manuscript submitted for publication). Similarly, these RRM also inhibited NF κ B-driven luciferase activity in transient transfection

studies performed in HeLa and PC3 cells. The apoptotic RRM α s are strong inhibitors of IKK activity in prostate and lung cancer cell lines (data not shown). This inhibition is a consequence of the direct binding of the RRM α s to IKK as evidenced by experiments showing that these RRM α s can inhibit active IKK *in vitro* (Fig. 2). In addition to the pharmacological inhibition of IKK by small molecules, inhibition of IKK by overexpression of an IKK dominant negative mutant (IKK β K44M) or a non-phosphorylatable form of I κ B α (I κ B α SS/AA) was sufficient for the reduction of cell viability in PC3 and A549 cells (Fig 3).

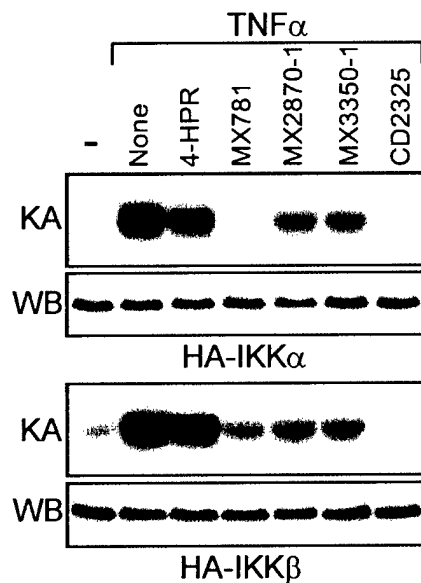


Figure 2. Inhibition of IKK activity *in vitro*. RRM α s inhibit activated IKK isolated from cancer cells. HeLa cells were transfected with HA-IKK α or HA-IKK β expression vectors and 16 hours post transfection were stimulated with TNF α for 10 min, after which the cells were lysed and HA-IKK α / β were immunoprecipitated with an anti-HA antibody. IKK α / β -containing Sepharose beads were incubated with 60 μ M of the indicated retinoids for 60 min in ice and then subjected to a kinase assay using GST-I κ B α (1-54) as substrate. Levels of IKK α / β were analyzed by western blot with an anti-HA antibody.

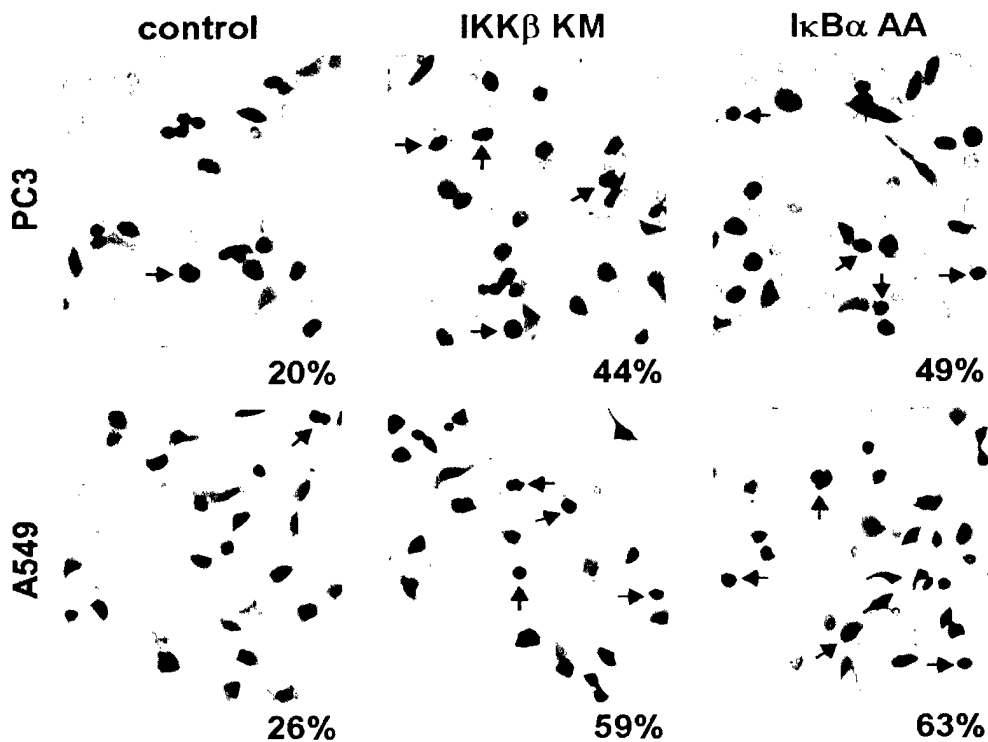


Figure 3. Inhibition of the IKK/NF κ B pathway reduces cell viability. PC3 and A549 cells were transiently transfected with empty vector (control) or mutants of IKK β (K44M) and I κ B α (S32/36A) together with a β -galactosidase expression vector. 24 hours after transfection, cells were double-stained with trypan blue and red-gal. Transfected cells (positive for β -galactosidase) and apoptotic cells (indicated by arrows) were counted in at least eight different fields (over 400 transfected cells) and the percentage of apoptotic cells is indicated below each panel. The experiment was repeated at least four times with very similar results, and data from one representative experiment are shown.

Inhibitors of IKK induce apoptosis in MDA-MB-468 cells. Based on the above observations and because of the high NF κ B activity levels reported in ER-negative BCCs, we examined the effect of RRM on IKK activity in MDA-MB-468 cells. Preincubation with MX781 but not RA completely inhibited the activation of IKK by TNF α . In contrast, other apoptotic RRM like MX3350-1, CD271, and CD2325 exerted a partial effect. As control, we examined the effect of known inhibitors of IKK that are not related to retinoids such as 15dPGJ2 and arsenite, which also elicited significant inhibition of IKK activity (Fig. 4A). These data correlated with cell proliferation studies. MX781 as well as the IKK inhibitors exerted a strong cell killing activity (Fig. 4B) and induced apoptosis as determined by Cell Death Detection ELISA (not shown). A striking correlation was observed between the inhibition of IKK and the induction of apoptosis by MX781, which indicated that this retinoid antagonist could induce apoptosis mainly through the inhibition of IKK. In contrast, the apoptotic molecules MX3350-1 and CD2325 also elicited a strong antiproliferative/cell killing activity, although only partially inhibited IKK, suggesting that inhibition of IKK/NF κ B pathways play a minimal role if any in the induction of apoptosis by this type of RRM. Other retinoids, such as 4-HPR, induced apoptosis in the absence of any effect on IKK, supporting that different retinoids can induce apoptosis through unique mechanisms of action.

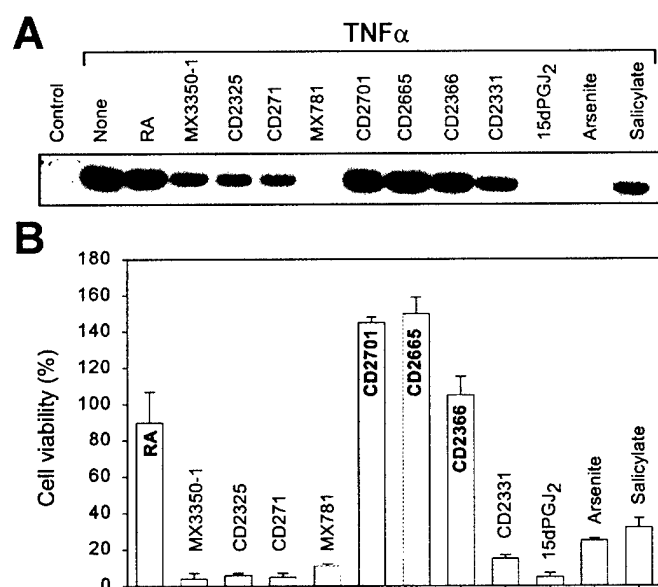


Figure 4. Inhibition of IKK correlates with induced cell death. (A) Effect of selective RRM and other small molecules on IKK activation. MDA-MB-468 cells were pre-incubated with 6 μ M of RA or the indicated RRM, 15 μ M 15dPGJ2, 100 μ M sodium arsenite or 20 mM salicylate for 4 hours prior to stimulation with 20 ng/ml of TNF α for 10 min. Subsequently, whole cell extracts were prepared and assayed for IKK activity using an immune complex kinase assay. (B) Effect of IKK inhibitors on cell proliferation. 3,000 cells were seeded in 96 well plates and treated with the indicated

compounds for 48 hours, when cell proliferation was measured by MTT. The percentage of cell viability was compared to control cells (100%) grown in the presence of solvent.

Problems encountered. We originally planned to perform transient transfection studies in BCCs to investigate the inhibition of NF κ B transcriptional activity by RRM. These experiments have been delayed because of technical difficulties to achieve high transfection efficiencies. The problems are being solved and we hope to finish the experiments in the near future. These transfection difficulties have also delayed experiments similar to those exposed in figure 3, which will be performed as soon as high transfection efficiencies are achieved. An extension of unexpended funds has been requested (and approved) to finalize these experiments during the coming months.

KEY RESEARCH ACCOMPLISHMENTS

- Novel selective RRM with unique structures and activities inhibit cell growth of ER-negative BCCs and induce apoptosis.
- The retinoid antagonist MX781 is a strong inhibitor of IKK in intact cells.
- Pharmacological inhibition of IKK inhibits cell proliferation and induces apoptosis in MDA-MB-468 cells.
- Inhibition of IKK/NF κ B signaling is sufficient to induce apoptosis.

REPORTABLE OUTCOMES

Funding applied:

Inhibitors of IKK/NF κ B as novel anticancer drugs. NIH. Submitted 06/01/2002.

Inhibitors of IKK as anticancer agents against breast cancer. CDMRP. Submitted 06/08/2002.

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Retinoids in combination therapies against breast cancer. University of California BCRP. Award # 8WB-0065 (07/01/2002-06/31/2004).

CONCLUSIONS

We have found that inhibitors of IKK induce apoptosis in breast cancer cells as well as in other tumor cells. This is an important finding in that this type of compound shows promise for the treatment and prevention of cancer, especially those that present constitutively high levels of IKK/NF κ B activity (including breast cancer cells). IKK inhibitors could be used as stand alone therapies, such as the selective RRM that are strong inducers of apoptosis, or could be useful in combination therapies to sensitize tumor cells to the anticancer efficiency of other therapies (radiation, drugs). Further experiments with these RRM in a pre-clinical setting, such as those reported by us in MDA-MB-468 xenografts, are necessary for the full understanding of the importance of this novel retinoid activity in the anticancer capacity of these compounds.

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